EXHIBIT 3

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Intranasal Immunization of Mice with a Mixture of the Pneumococcal Proteins PsaA and PspA Is Highly Protective against Nasopharyngeal Carriage of *Streptococcus pneumoniae*

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Acquisition of pneumococci is generally from carriers rather than from infected individuals. Therefore, to induce herd immunity against *Streptococcus pneumoniae* it will be necessary to elicit protection against carriage. Capsular polysaccharide-protein conjugates, PspA, and PsaA are known to elicit some protection against nasopharyngeal carriage of pneumococci but do not always completely eliminate carriage. In this study, we observed that PsaA elicited better protection than did PspA against carriage. Pneumolysin elicited no protection against carriage. Immunization with a mixture of PsaA and PspA elicited the best protection against carriage. These results indicate that PspA and PsaA may be useful for the elicitation of herd immunity in humans. As PspA and pneumolysin are known to elicit immunity to bacteremia and pneumonia, their inclusion in a mucosal vaccine may enable such a vaccine to prevent invasive disease as well as carriage.

Infections with *Streptococcus pneumoniae* are a major cause of otitis media, meningitis, and fatal pneumonia worldwide (12). In the developing world, pneumococci cause over one million fatal respiratory infections per year in young children and infants (17, 24). Although the polysaccharide-protein conjugate vaccine presently under development appears to be efficacious against bacteremic infections with pneumococci, it will not protect against capsular types not included among the conjugates comprising the vaccine (S. Black, H. Shinefield, P. Ray, L. Edwi, B. Fireman, T. K. P. V. S. Group, R. Auystrian, G. Siber, J. Hackell, K. Robert, and I. Chang, Late Breaker Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. LB-9, 1998) and the vaccine is likely to be too expensive for widespread use in the developing world. Although the polysaccharide-protein conjugate vaccines elicit modest protection against carriage, they have not been reported to eliminate carriage in humans (27) or in a mouse model (38). Moreover, there is evidence that reduction in nasopharyngeal carriage of serotypes included in the conjugate vaccine may be offset by an increase in carriage of potentially invasive nonvaccine serotypes (27). To provide adequate herd immunity and widespread protection in the developing world, a new, or modified, vaccine will be needed. One possibility is to use protection-eliciting cross-reactive pneumococcal proteins as immunogens (9, 29).

Several pneumococcal proteins are known to elicit protective immunity. PspA has been shown to inhibit complement fixation in vivo and in vitro (1, 25, 37) and to elicit protection against fatal bacteremia and sepsis caused by a broad spectrum of pneumococcus serotypes (20, 22, 36). Pneumolysin interferes with host immunity and inflammatory responses by a variety of functions that include complement fixation at inappropriate sites and inhibition of appropriate phagocyte functions (3, 4, 28). Pneumolysin also inhibits ciliary activity in the bronchus and is important in the pathogenesis of pulmonary infections (1a, 16, 28).

Intranasal (i.n.) immunization of mice with either PspA or polysaccharide-protein conjugates has been shown to elicit measurable protection against carriage of *S. pneumoniae* (38). PspA has also been shown to elicit protection against otherwise fatal intravenous, i.n., or intratracheal inoculation with pneumococci (38). PsaA has also been shown to elicit protection against carriage (B. K. De, J. S. Sampson, E. W. Ades, R. C. Huebner, D. L. Jue, S. E. Johnson, M. Espina, A. R. Stinson, D. E. Briles, and G. M. Carlone, submitted for publication). It is the product of a gene in an ABC transporter operon thought to be involved in the transport of manganese into pneumococci (15). Mutations that block expression of PsaA do not affect growth of pneumococci in vitro but completely eliminate virulence (5). Although PsaA has been shown to elicit protection against fatal bacteremia (35), its protection against sepsis is not as robust as that elicited by PspA (D. E. Briles, unpublished data).

The present study compares the abilities of PspA and PsaA to elicit protection against carriage and examines the possibility that an immunization combining both PspA and PsaA might elicit even better protection against carriage. The results obtained with PspA and PsaA were also compared to those obtained with pneumolysin, since the latter protein has been found to be especially important in the pathogenesis of pulmonary infections (28). A relatively nontoxic variant of pneu-

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TABLE 1. Serum and salivary antibody responses to PspA, PsaA, and PdB[a]

Antibody		No. of mice[b]	Immunogens					
Source	Specificity		CTB only	rPspA + CTB	rPsaA + CTB	rPdB + CTB	rPspA + rPsaA + CTB	rPspA + rPdB + CTB[c]
Serum	PspA	12	<0.002	30.9 (1.4)	<0.002	<0.002	32.4 (1.2)	10.5 (1.2)
Serum	PsaA	12	<0.034	<0.034	257 (1.15)	<0.034	234 (1.2)	<0.034
Serum	PdB	8	<0.0009	<0.0009	<0.0009	2.15 (1.1)	<0.0009	2.45 (1.3)
Saliva	PspA	12	<0.002	0.014 (1.8)	<0.002	<0.001	0.008 (1.2)	ND[d]
Saliva	PsaA	12	<0.034	<0.034	1.00 (1.1)	<0.016	1.34 (1.1)	<0.016
Saliva	PdB	8	<0.009	<0.0009	<0.0009	0.0019 (1.1)	<0.0009	0.0018 (1.1)

[a] In all cases, $P \leq 0.0004$ for immune versus nonimmune controls. Data is expressed as the geometric means of micrograms of immunoglobulin per milliliter (standard error factors are shown in parentheses).
[b] Number in each group in the indicated row except for the last column.
[c] There were eight mice in all groups in this column.
[d] ND, not determined.

molysin, PdB, has been shown to elicit protection against fatal sepsis (2, 30).

MATERIALS AND METHODS

Antigens, immunization, and challenge. Recombinant lipidated PsaA was prepared as has been previously described by the expression in *Escherichia coli* of an 830-bp fragment of pneumococcal DNA from the *psaA* gene of the capsular-type 6B strain. The recombinant PsaA protein is fused at the amino terminus to the 51-bp signal sequence of *Borrelia burgdorferi* outer membrane surface protein encoded by *ospA* (De et al., submitted). Recombinant PspA/Rx1 (amino acids 1 to 302) was isolated by nickel affinity chromatography from *E. coli* BL21(DE3) carrying pUAB055. To construct pUAB055, a 909-bp fragment of *pspA* from pneumococcal strain Rx1 was cloned into the pET20b vector of Novagen, Inc. Between the *Nco*I and *Xho*I sites. Recombinant PspA/Rx1 contains the first 302 amino acids of mature PspA plus six polyhistidines added through protein fusion at the C-terminal end. Recombinant protein was isolated by nickel affinity chromatography as described in the Novagen manual with the exception that elution from the column was performed in 60 mM imidazole buffer. The isolation of the recombinant nontoxic pneumolysin (PdB) used in these studies has been described previously (30).

Immunization was i.n. in a 10-μl volume as previously described (10). In an attempt to compensate for differences in the immunogenicity of these proteins, mice were immunized i.n. with 0.5 μg of PspA, 0.5 μg of PsaA, or 10 μg of pneumolysin. Other mice received mixtures of two of the proteins at the same concentrations listed above. PsaA is a lipoprotein and is immunogenic with i.n. immunization without the need for an adjuvant (De et al., submitted). PspA and pneumolysin are not lipidated, and PspA requires an adjuvant such as the cholera toxin or the cholera toxin B subunit (CTB) to elicit immunity (38, 40). As pneumolysin is less immunogenic than PspA when administered parenterally, we made the assumption that it should also be administered with adjuvant when given i.n. To keep the immunization protocols as similar as possible, CTB (List Biological Laboratories, Campbell, Calif.) (4 μg/immunization) was given with each immunization regardless of the antigen(s) given. CTB contains about 0.1% (weight/weight) intact cholera toxin.

These studies were conducted with *S. pneumoniae* L82016 and E134, strains which are carried on nasal tissues in high numbers following i.n. inoculation. These strains are capsular types 6B and 23, respectively. They are both readily cleared from the circulation when injected intravenously at doses of 10^6 or fewer bacteria. Consistent with their low virulence in the blood, nasal carriage of these strains does not lead to bacteremia or sepsis. By choosing strains that do not readily cause sepsis in mice under these conditions, we can be sure that any pneumococci present in the nasal wash were there due to carriage and not due to generalized sepsis. Therefore, protection resulting in a reduction in the numbers of pneumococci in the nose would be a result of immunity to carriage and not an indirect effect of protection against bacteremia and sepsis.

From preliminary studies, we determined that the immune response to i.n. immunization was much smaller if the immunizations were administered farther than 1 week apart (data not shown). In the present studies, mice were immunized with antigen on the Mondays and Fridays of 3 consecutive weeks. These immunizations included CTB for the first 2 weeks of immunization. During the last week (i.e., the last two immunizations), mice received antigen alone. Control mice received only CTB for the first 2 weeks and only saline for the last week. After a 3-week rest, all mice were challenged i.n. with either 4.7×10^6 CFU of L82016 or 10^6 CFU of E134. L82016 is a type 6B pneumococcus used in previously published carriage studies (38, 39). L82016 was originally isolated from the blood of a pneumonia patient in Alaska. E134 is a capsular type 23 pneumococcus isolated from the blood of a 47-year-old female patient at the University of Alabama at Birmingham Hospital with pneumococcal pneumonia. L82016 has a clade 2, family 1 PspA, and E134 has a clade 1, family 1 PspA. The Rx1 PspA used for immunization is clade 2, family 1. Previous studies have shown that there is very effective cross-protection against sepsis in mice among the clade 1 and clade 2 proteins of family 1 (D. E. Briles, unpublished data).

Although the number of CFU used for inoculation is high, it should be pointed out that in earlier studies we demonstrated that doses of L82016 as small as 2,000 CFU could establish carriage in the majority of the mice, and the number of CFU carried in each mouse was about the same as when 10^7 CFU were administered. The only difference was that the higher inoculum doses resulted in carriage in all, rather than most, of the challenged mice (39). Doses of 10^7 CFU occasionally resulted in death, thus defining the upper limit of the useful dose in a carriage study.

Immune responses and levels of carriage and bacteremia. Saliva was collected from all mice 14 days after the last immunization by standard enzyme-linked immunosorbant assay procedures, where the microtitration plates were coated with the same recombinant proteins used for immunization (33). Serum was collected 17 days after the last immunization from a 75-μl retroorbital bleed (13), and the mice were challenged 18 days after the last immunization. Six days after challenge (42 days after the initial immunization), the pneumococci were washed from mouse nasal passages and were enumerated by plating on modified blood agar as described previously (38, 39). Antibody levels were determined by using an antiimmunoglobulin serum as previously described (38), except that pooled mouse serum containing a standardized amount of antibody to PspA was used as the standard for all three assays. The pooled serum was itself standardized by using purified monoclonal antibody to PspA (21). Thus, the optical density (OD) of antibody to PsaA or pneumolysin was assumed to have the same amount of antibody as an identical OD of antibody to PspA.

Statistics. Statistical comparisons of antibody levels and CFU levels were all carried out on log transformed data by the Wilcoxon and/or Student's *t* tests. The levels of antibodies to PsaA, PspA, and PdB were expressed as the geometric mean of antibody in micrograms with the standard error factor. The standard error factor is that number by which the geometric mean must be multiplied and divided to obtain, respectively, the upper and lower bounds of standard error. CFU data were expressed as average $\log_{10}$ CFU ± the standard error factor. The lower limit of CFU detected from nasal wash was 60 CFU. Mice with no detectable CFU were assigned a value of 45 CFU for the purposes of calculations.

RESULTS

All three immunogens elicited antibody in the serum and saliva (Table 1). The highest titers were those to PsaA, which were almost 10-fold greater than serum antibody levels to PspA and almost 100-fold higher than mucosal antibody levels to PdB. The levels of antibodies to PsaA and PspA were higher than the levels of antibody to PdB, even though the mice were given 1/20th as much PsaA or PspA as PdB.

With both challenge strains, L82016 and E134, fewer pneumococci were recovered from mice immunized with PspA than from those given CTB alone (Tables 2 and 3). The effect of PspA immunization on carriage was significant, however, only for L82016. Immunization with PsaA resulted in a >10-fold decrease in E134 carriage and a >100-fold decrease in L82016 carriage. In each case, the protection afforded by immunization with PsaA was highly significant. In contrast to the results with PsaA and PspA, immunization with PdB had no observable effect on nasopharyngeal carriage.

We also examined the effects on carriage of immunization

TABLE 2. Effect of immunization with pneumococcal proteins on nasal carriage of L82016

Immunogen(s)	No. of mice	CFU/nasal wash (mean log [standard error])	*P* value of immunogen(s) versus CTB only	
			Student's *t* test	Wilcoxon test
CTB only	11	4.48 (0.21)		
PspA	10	3.36 (0.34)	0.01	0.013
PsaA	10	2.14 (0.25)	<0.0001	<0.0001
PdB	4	5.22 (0.09)	0.64	0.078
PspA + PsaA	10	1.70 (0.08)[a]	<0.001	<0.0001
PspA + PdB	4	5.10 (0.22)	0.14	0.18

[a] Seven of the 10 mice in this group had undetectable levels of CFUs (<60 CFU per nasal wash).

with combinations of PspA and either PsaA or PdB. For both challenge strains, immunization with the mixture of PspA and PdB gave no greater protection against colonization than was achieved by immunization with PspA alone. Immunization with the mixture of PspA and PsaA, however, resulted in less carriage of L82016 or E134 than did immunization with either PspA or PsaA alone. In each case, the protection elicited by the mixture of PsaA and PspA was significantly better than that elicited by PsaA or PspA alone ($P < 0.05$ by both Student's *t* test and the Wilcoxon test). In the case of L82016, the reduction in the number of CFU resulting from immunization with the PsaA-PspA mixture was almost 1,000-fold. In the case of E134, the reduction was about 300-fold. It is noteworthy that the mixture of PsaA and PspA resulted in greater protection against carriage with E134 than did immunization with PsaA. This synergistic effect of the mixture was observed even though PspA alone resulted in no statistically significant protection against strain E134.

DISCUSSION

This study demonstrated that immunization with both PspA and PsaA, compared to immunization with either protein alone, provided better protection against nasal colonization by pneumococci. This observation suggests that these two molecules play different, and additive, roles in the virulence of pneumococci.

Somewhat surprisingly, the recombinant PspA used in this study was observed to elicit significantly less protection against carriage with L82016 than had been observed in an earlier study in which full-length PspA (about 600 amino acids) was used as the immunogen. There are several possible explanations for this difference in results. The full-length molecule may have important epitopes in the proline-rich and/or choline-binding regions that elicit immunity against carriage but which are absent in the recombinant (amino acids 1 to 302) PspA fragment used here. In the earlier studies, the immunizing PspA was isolated from L82016, so it could also have had important L82016 epitopes lacking in the heterologous rRx1 PspA used here. Finally, although PspA isolated from pneumococci is at least 95% pure (8), it probably contains small amounts of other pneumococcal antigens, such as PspC (7, 11) (also called SpsA and CbpA [18, 31]), which is also thought to be involved in adherence and carriage (31).

PdB was not observed to elicit protection against carriage, even though pneumolysin has been shown to play an important role in pulmonary and systemic infections, and immunity against PdB can elicit protection against fatal challenge by either the i.n. or the intraperitoneal route (2). These results indicate that, with respect to anti-PdB-mediated protection, there must be critical differences between the pneumococcal airway environment in the lung and the mucosal surfaces of the nose. The failure of immunization with PdB to prevent carriage is also consistent with studies using defined, pneumolysin-negative pneumococci, which colonized the nasopharynx of mice as efficiently as did the wild-type parent (32).

In terms of the relative abilities of immunities to the three antigens to protect against carriage, we must bear in mind that much higher levels of antibody were observed in secretions from mice immunized with PsaA than were observed in the secretions of mice immunized with PspA or PdB. Thus, these results do not rule out the possibility that comparable levels of mucosal immunity to PspA or PdB might have elicited as much or more protection against carriage than did PsaA.

The question of how PsaA and PspA elicit protection against carriage remains unanswered. PspA is able to interfere with complement fixation (25, 37), which is important for virulence in invasive models of disease (23). Whether PspA-mediated inhibition of complement plays a role in carriage is not known. We have shown that although PspA can enhance carriage, it is not necessary for carriage (D. E. Briles and A. Virolainen, unpublished data). Thus, PspA most likely does not act as an essential adhesin.

Although PspAs are serologically variable, they can be divided into families (S. K. Hollingshead, R. S. Becker, and D. E. Briles, submitted for publication) which contain highly cross-reactive members (S. K. Hollingshead and D. E. Briles, unpublished data). Strains L82016 and E134 both express PspAs of family 1, the same family as the recombinant Rx1 PspA (S. K. Hollingshead et al., submitted) used for immunization. Family 1 can be further divided into clades 1 and 2 based on their sequence (S. K. Hollingshead et al., submitted), but the proteins in the two clades are so cross-reactive that they cannot be reliably distinguished serologically (S. K. Hollingshead and D. E. Briles, unpublished data).

PsaA is a member of a family of metal-binding lipoproteins and is part of an ABC transporter complex with specificity for manganese and possibly zinc (15). The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein (19). The presence of PsaA is necessary for carriage, but as with PspA, the precise mechanism by which it contributes to carriage is not known. One possibility is that PsaA is required to scavenge the Mn^{2+} needed for growth in the nasopharynx; alternatively, Mn^{2+} transported into the cell may act as a signal, resulting in the expression of candidate adhesins such as PspC/CbpA (26).

The best immunization route for elicitation of immunity to carriage of pneumococci is not known with certainty. In the case of *Haemophilus influenzae*, it is clear that intramuscular

TABLE 3. Effect of immunization with pneumococcal proteins on nasal carriage of E134

Immunogens	No. of mice	CFU/nasal wash (mean log [standard error])	*P* value of immunogen(s) versus CTB only	
			Student's *t* test	Wilcoxon test
CTB only	11	4.94 (0.30)		
PspA	10	4.32 (0.42)	0.24	0.43
PsaA	10	3.51 (0.36)	0.0061	0.0062
PdB	4	4.64 (0.40)	0.60	0.75
PspA + PsaA	10	2.45 (0.31)	<0.0001	<0.0001
PspA + PdB	4	4.16 (0.37)	0.18	0.23

immunization of children with polysaccharide-protein conjugates elicits protection against both bacteremia and carriage. In the case of pneumococci, it is possible that optimal immunization against carriage needs to be carried out mucosally. Immunization with conjugate vaccines fails to elicit complete protection against carriage in children (14, 27). In animal studies, it has been shown that subcutaneous immunization with PspA is less able to elicit protection against carriage than is mucosal immunization, even though higher serum antibody titers were elicited by subcutaneous rather than mucosal immunization (38). Preliminary studies with PsaA, however, have demonstrated that parenteral immunization can elicit some immunity to carriage (De et al., submitted).

If mixtures of PsaA and PspA are found to be protective against carriage with most, or all, pneumococcal strains then these antigens could be added to other pneumococcal vaccines (9, 12, 34) to improve their ability to confer herd immunity. Mucosal immunization with pneumococcal proteins can also elicit significant serum antibody responses (38, 41). Moreover, both PspA and PdB have been shown to elicit immunity to bacteremia, sepsis, and/or lung infections (6, 29) (D. E. Briles and J. C. Paton, unpublished data). It is possible, therefore, that a mucosal vaccine containing PsaA, PspA, and possibly PdB could be a stand-alone vaccine that could elicit protection against systemic infection as well as carriage.

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